

## Supporting document 1

Safety assessment – Application A1094 (at Approval)

Food derived from Herbicide-tolerant Cotton Line DAS-81910-7

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## Summary and Conclusions

### Background

A genetically modified (GM) cotton line with OECD Unique Identifier DAS-81910-7 hereafter also referred to as cotton line 81910, has been developed that is tolerant to two herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), and glufosinate ammonium. Tolerance to 2,4-D is achieved through expression of the enzyme aryloxyalkanoatedioxygenase-12 (AAD-12) encoded by the *aad-12* gene derived from the soil bacterium *Delftia acidovorans*. Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat* gene derived from another soil bacterium *Streptomyces viridochromogenes*.

In conducting a safety assessment of food derived from cotton line 81910, a number of criteria have been addressed including: characterisation of the transferred genes including their origin, function and stability in the cotton genome; the nature of the introduced proteins and their potential to be either allergenic or toxic in humans; compositional analyses and any resultant changes in the whole food. This approach evaluates the intended and any unintended changes in the plant.

This safety assessment report addresses food safety and nutritional issues. It therefore does not address:

- any risks to the environment that may occur as the result of growing GM plants used in food production
- any risks to animals that may consume feed derived from GM plants
- the safety *per se* of food derived from the non-GM conventional crop plant.

Food derived from the non-GM (conventional) plant with an accepted history of safe use is used as the benchmark for the comparative analysis.

### History of Use

The host organism is cultivated cotton (*Gossypium hirsutum* L.). Cotton is one of the oldest cultivated crops and is grown worldwide primarily as a fibre crop but also as a source of food products derived from the seed. Such products need to be highly processed because of the presence of natural toxicants (gossypol) and anti-nutrients (cyclopropenoid fatty acids) in unprocessed cottonseed. The main food products include cottonseed oil and linters.

## **Molecular Characterisation**

Cotton line 81910 contains two genes derived from bacteria that were introduced on a single expression cassette via *Agrobacterium*-mediated transformation. The *aad-12* gene from *Delftia acidovorans* encodes the AAD-12 protein, an  $\alpha$ -ketoglutarate-dependent dioxygenase that inactivates 2,4-D. The *bar* gene from *Streptomyces hygrosopicus* encodes phosphinothricin N-acetyltransferase (PAT), an enzyme conferring tolerance to herbicides containing glufosinate ammonium (phosphinothricin). Detailed molecular analyses of cotton line 81910 indicate that one complete copy of the two-gene expression cassette is present at a single insertion site. Plasmid backbone analysis shows no extraneous sequences, including antibiotic resistance genes derived from the plasmid, were transferred to the cotton line 81910 genome.

The introduced genetic elements and the expression of new proteins in cotton line 81910 were shown by phenotypic analysis and molecular techniques to be stably inherited from one generation to the next across multiple generations. The pattern of inheritance supports the conclusion that the herbicide-tolerance traits occur within a single locus in the cotton line 81910 genome and are inherited in accordance with Mendelian principles.

## **Characterisation of Novel Proteins**

A range of characterisation studies confirmed the identity of AAD-12 and PAT derived from cotton line 81910 and also their equivalence with the corresponding protein produced in a bacterial expression system. The plant AAD-12 and PAT proteins have the expected molecular weight (32 kDa and 20 kDa respectively), immunoreactivity, lack of glycosylation, amino acid sequence and enzyme activity.

Both AAD-12 and PAT were detected in all plant parts analysed. AAD-12 is present in highest concentration in 4-leaf stage leaves and pollen and lowest in the roots. For PAT, the highest level was in the 4-leaf leaves and the lowest in pollen. The mean level of AAD-12 in seed from cotton line 81910 is approximately 19  $\mu\text{g/g dw}$  (0.0019%) and that of PAT is approximately 4  $\mu\text{g/g dw}$  (0.0004%). As the mean percent dry weight of total protein in cottonseed from cotton line 81910 is approximately 25% dw, the amount of AAD-12 protein in cotton line 81910 is calculated to be 0.008% of total protein and that of PAT to be 0.002%.

## **Herbicide Metabolites**

In the case of cotton line 81910 the herbicide residues resulting from the spraying of 2,4-D and glufosinate ammonium have been assessed in previous applications (see e.g. Application A 1046). There are no concerns that the spraying of cotton line 81910 with 2,4-D or glufosinate ammonium would result in the production of any novel metabolites that have not been previously considered.

## **Compositional Analyses**

Detailed compositional analyses on seed from cotton line 81910, the control 'Coker 310' and six commercial varieties were conducted on plants grown under normal agricultural conditions at eight trial sites in cotton growing regions of the U.S. The analyses included proximates (protein, fat, ash, moisture, carbohydrates by calculation), fibre components, fatty acids, amino acids, minerals, vitamins and anti-nutrients. The levels of 59 of these key analytes in cotton line 81910 were compared to those in the control and to the ranges found in commercial non-GM cotton varieties grown concurrently in the same trial, or as reported in the literature.

For seven of the analytes statistically significant differences were found between seeds from cotton line 81910 and the control, however all differences were small in magnitude and were within the range established for existing commercial cotton varieties. The composition of cotton can vary significantly with the site and the prevailing agricultural conditions, and the differences reported are attributable to normal biological variation. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in seed from cotton line 81910 when compared with levels found in seed of conventional cotton varieties available on the market.

## **Conclusion**

No potential public health and safety concerns have been identified in the assessment of cotton line DAS-81910-7. On the basis of the data required from the Applicant, and other available information, food derived from herbicide-tolerant cotton line DAS-81910-7 is as safe for human consumption as food derived from conventional cotton varieties.

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## List of Abbreviations

AAD-12	aryloxyalkanoatedioxygenase-12
ADF	acid detergent fibre
ae	acid equivalent (refers to herbicide)
ai	active ingredient (refers to herbicide)
<i>bar</i>	<i>bialaphos resistance gene</i>
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CsVMV	Cassava vein mosaic virus
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
T-DNA	transfer DNA
dw	dry weight
EFSA	European Food Safety Authority
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FDR	False discovery rate
FSANZ	Food Standards Australia New Zealand
fw	fresh weight
GM	genetically modified
ILSI	International Life Sciences Institute
kb	kilobase
kDa	kilo Dalton
LC/MS	high performance liquid chromatography/electrospray mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
MAR	matrix attachment region
NDF	neutral detergent fibre
NS	not significant
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
P	Probability
<i>pat</i>	<i>phosphinothricin N-acetyltransferase gene</i>
PCR	polymerase chain reaction
ppb	parts per billion
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
U.S.	United States of America
WHO	World Health Organisation

# 1. Introduction

A genetically modified (GM) cotton line with OECD Unique Identifier DAS-81910-7 (hereafter also referred to as cotton line 81910) has been developed for tolerance to herbicides of the aryloxyalkanoate family, specifically the phenoxy auxin 2,4-dichlorophenoxyacetic acid (2,4-D), and to the herbicide glufosinate ammonium.

Tolerance to 2,4-D is achieved through expression of the enzyme aryloxyalkanoatedioxygenase-12 (AAD-12) encoded by the *aad-12* gene derived from *Delftia acidovorans*, a gram-negative soil bacterium. The AAD-12 protein has previously been assessed by FSANZ in two soybean lines (FSANZ, 2011; FSANZ, 2013a; Wright *et al.*, 2010). Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat* gene derived from another soil bacterium *Streptomyces viridochromogenes*. This protein has been considered in 18 previous FSANZ approvals and globally is represented in six major crop species and over 30 approved GM single plant events (FAO GM Foods Platform - <http://www.fao.org/food/food-safety-quality/gm-foods-platform/browse-information-by/commodity/en/>).

It is anticipated that cotton line 81910 will be grown predominantly in the United States of America (U.S.) subject to approval. The Applicant has stated there is currently no intention to grow the line in Australia or New Zealand.

## 2. History of use

### 2.1 Host organism

The host organism is cultivated cotton (*Gossypium hirsutum* L.). Cotton is one of the oldest cultivated crops and is grown primarily as a fibre crop, providing approximately 35% of the textile fibre used in the world ([http://www.agmrc.org/commodities\\_products/fiber/cotton-profile/](http://www.agmrc.org/commodities_products/fiber/cotton-profile/)). Only the cotton boll, which develops from the plant ovary, is used for either textile fibre or food/feed. The cotton boll, once harvested, is processed ('ginned') to separate the fibre from the cottonseed.

Cottonseed is processed into four major by-products: oil, meal, hulls and linters (see Figure 1), of which the oil and linters are typically used as human food. By weight, processing of cottonseed typically yields 16% oil, 45% meal, 26% hulls and 9% linters, with 4% lost during processing (Cherry, 1983). Food products are limited to highly processed products because of the presence of natural toxicants (gossypol) and anti-nutrients (cyclopropenoid fatty acids) in unprocessed cottonseed. Processed cottonseed oil has been used safely for human food for over a century. Meal and hulls are mainly used as livestock feed.

The fatty acid profile of cottonseed oil comprises 70% unsaturated fatty acids (including 52% linoleic and 18% oleic) and 26% saturated (mainly palmitic). The natural oil has a strong and unpleasant flavour and requires a deodorisation process to render it palatable. Cottonseed oil has a variety of food uses including frying oil, salad and cooking oil, and inclusion in mayonnaise, salad dressing, shortening, and margarine. In the course of processing to food grade quality oil, proteins are destroyed by high temperatures and pressure, or are separated out by extraction with a non-polar solvent. Subsequent alkali treatment and deodorisation steps are likely to remove any last detectable traces of protein in the refined oil. Deodorisation also greatly reduces the cyclopropenoid fatty acid content.

Cotton linters are short fibres that remain after the long fibres have been removed at the ginning process for textile manufacture.

Linters consist of nearly pure (> 99%) cellulose and are used in both chemical and high fibre dietary products. Food uses include casings for processed meats, and as a viscosity enhancer (thickener) in ice cream, salad dressings and toothpaste.

Another possible food product that can be derived from the cotton plant is bee pollen (Krell, 1996).

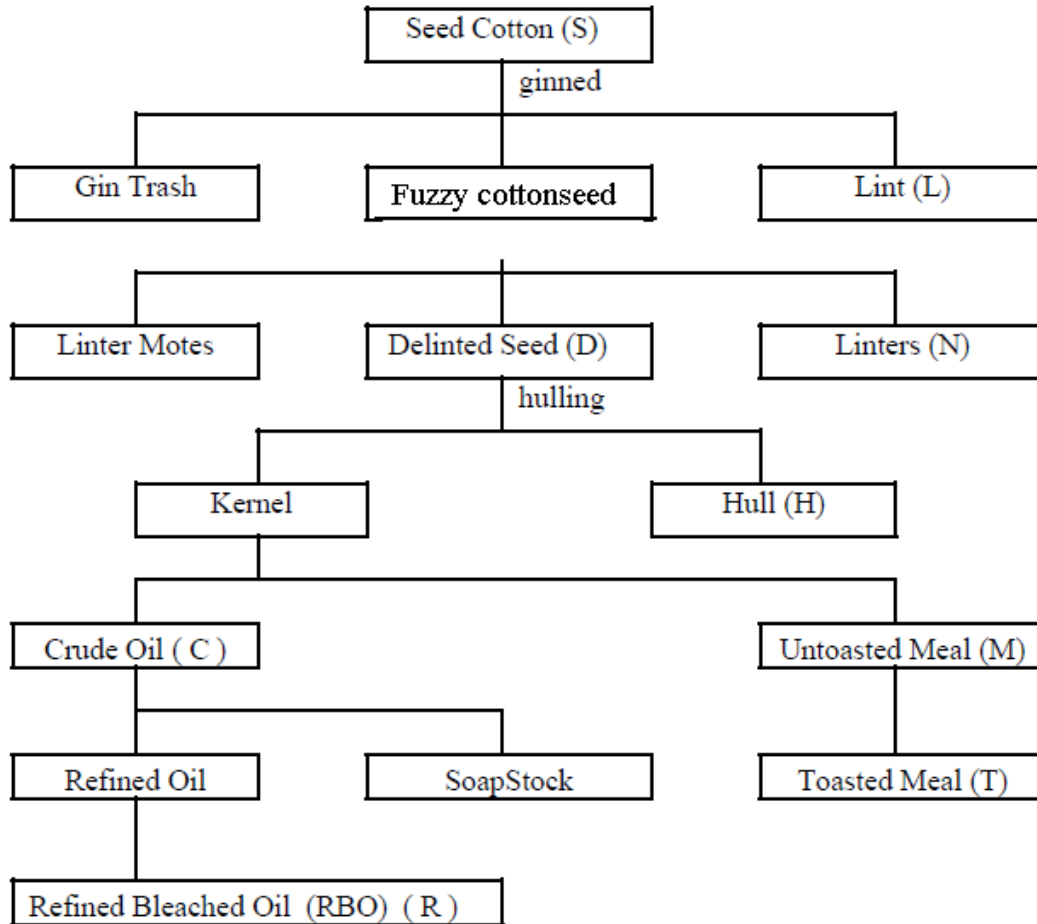


Figure 1: The major processed fractions obtained from cottonseed

The material left after extraction of crude cottonseed oil is cottonseed meal. This product is not used for human consumption in Australia or New Zealand. Cottonseed meal is permitted to be used for human food (after processing) in the U.S. and other countries, but is primarily sold for stock feed. The levels of gossypol in the meal after extraction are reduced by approximately half.

Cotton is not grown in New Zealand. Australia has significant plantings of the crop although the area varies annually due largely to prevailing environmental factors. GM varieties now represent almost 100 per cent of cotton grown across the country and all traits are for protection against insect pests and/or tolerance to a herbicide (ABCA, 2012).

Although fibre is seen as the main product, cotton is also Australia's major oilseed crop. Most cottonseed is exported as fuzzy seed<sup>1</sup> destined for animal feedlots but a proportion of the seed is retained to produce oil, mainly for domestic use.

<sup>1</sup> Fuzzy (or whole) cottonseed is the linted cottonseed remaining after the ginning process which removes fibres for textile production (refer to Figure 1).



In 2012, some 190,000 tonnes of oil was produced in Australia (FAOSTAT – available at <http://faostat3.fao.org/faostat-gateway/go/to/home/E>).

The cotton cultivar ‘Coker 310’ was used as the parental variety for the genetic modification described in this Application, and thus is regarded as the near-isogenic line for the purposes of the comparative assessment. It is not grown commercially in Australia. ‘Coker 310’ was developed by the cotton division of Coker’s Pedigreed Seed Company and is an older commercial variety of upland cotton generated from a cross of ‘Coker 100 Staple’ and ‘Deltapine 15’ and selected through successive generations of line selection (Bowman *et al.*, 2006; Smith *et al.*, 1999). ‘Coker’ cultivars are U.S. cultivars that are widely used to produce GM cotton lines because they can be readily cultured and regenerated in the laboratory (OGTR, 2008). Traits introduced into ‘Coker’ cultivars are transferred to commercial cultivars by backcrossing.

## 2.2 Donor organisms

### 2.2.1 *Delftia acidovorans*

The *aad-12* gene was sourced from the bacterial species *Delftia acidovorans* strain MC1, a strain isolated from herbicide-contaminated building rubble (Müller *et al.*, 1999). This bacterium is a member of the Pseudomonads, a ubiquitous group of environmental gram negative bacteria. It was originally classified in the genus *Pseudomonas*, then renamed in the genus *Comamonas* (Tamaoka *et al.*, 1987) and finally renamed again to *Delftia* (Wen *et al.*, 1999).

*Delftia* spp. are aggressive colonisers of the rhizosphere of various crop plants and have a broad spectrum of antagonistic activity against plant pathogens (see e.g. El-Banna, 2007; Han *et al.*, 2005). They have also been found to possess a variety of biodegradation mechanisms that could be exploited in the development of systems for the removal of chemicals that may be released into the environment (Müller *et al.*, 1999; Patel *et al.*, 1998; Urata *et al.*, 2004). On rare occasions, *Delftia* spp. have been implicated in human infections (see e.g. Chun *et al.*, 2009 and references therein).

*D. acidovorans* is one of several microorganisms that has been proposed as a bioconverter for use in the food industry to transform ferulic acid into vanillin and related flavour metabolites (Labuda *et al.*, 1992). However, commercial application has not been realised (see e.g. Yoon *et al.*, 2005). The polyhydroxyalkanoates produced by *D. acidovorans* and other bacteria have been proposed for use as biomaterial for use in tissue engineering and other medical applications (Sudesh, 2004).

### 2.2.2 *Streptomyces viridochromogenes*

The source of the *pat* gene is the bacterial species *Streptomyces viridochromogenes*, strain Tü494 (Wohlleben *et al.*, 1988). The *Streptomycetaceae* bacteria were first described in the early 1900’s. These organisms are generally soil-borne, although they may also be isolated from water. They are not typically pathogenic to animals including humans, and few species have been shown to be phytopathogenic (Bradbury, 1986; Kützner, 1981).

Although these organisms are not used in the food industry, the *pat* gene from *S. viridochromogenes*, has been used to confer glufosinate ammonium-tolerance in a range of food producing crops. The *bar* gene from the closely related *S. hygrosopicus* produces a protein that is structurally and functionally equivalent to the protein encoded by the *pat* gene (Wehrmann *et al.*, 1996) and has similarly been used widely for genetic modification of crop species.

### 2.2.3 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of cotton line 81910 (refer to Table 1). These non-coding sequences are used to drive, enhance or terminate expression of the two novel genes. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not pathogenic in themselves and do not cause pathogenic symptoms in cotton line 81910.

## 3. Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome;
- a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation process itself;
- the genetic stability of the inserted DNA and expressed traits.

#### Studies submitted:

Mo, J.; Ring, S. (2012). Molecular characterization of DAS-81910-7 cotton. Study ID: 120456 Dow AgroSciences, LLC. Indianapolis, IN. (unpublished).

Mo, J.; Cruse, J. (2013). Cloning and characterization of the DNA sequence for the insert and its flanking border regions of DAS-81910-7 cotton. Study ID: 110752 Dow AgroSciences, LLC. Indianapolis, IN. (unpublished).

Guttikonda, S.K. (2013). Bioinformatics evaluation of the putative reading frames across the whole T-DNA insert and junctions in DAS-81910-7 cotton for potential protein allergenicity and toxicity. Study ID: 130619 Dow AgroSciences, LLC. Indianapolis, IN. (unpublished).

Gao, Z.; Ring, S.; Guttikonda, S.; Cruse, J.K. (2013). Cloning and analysis of the DNA sequence from DAS-81910-7 cotton insertion site in Coker 310 cotton. Study ID 130924 Dow AgroSciences, LLC. Indianapolis, IN. (unpublished).

Rapier, K. (2012). Molecular characterization of DAS-81910-7 cotton within a single segregating generation. Study ID: 120457 Dow AgroSciences, LLC. Indianapolis, IN. (unpublished).

### 3.1 Method used in the transformation

Cotton cultivar 'Coker 310' was transformed using an *Agrobacterium*-mediated transformation procedure based on that of Umbeck (1992). Hypocotyl segments from germinated seedlings were co-cultured with *Agrobacterium* strain LBA4404 carrying plasmid vector pDAB4468 (see Figure 2) and then placed on callus-inducing media containing carbenicillin to inhibit the growth of excess *Agrobacterium*, and glufosinate to inhibit growth of untransformed cells.

Calli containing the two genes of interest (*aad-12* and *pat*) were selected by gene-specific polymerase chain reaction (PCR) and transferred to regeneration medium. The subsequent rooted plants (T<sub>0</sub>) were then transferred to a glasshouse. As a further screen, putative transformants were selected by painting leaves with glufosinate ammonium and were then sampled for molecular analysis that included verification of the absence of vector backbone and presence of the *aad-12* and *pat* genes.

Selected T<sub>0</sub> plants were self-pollinated to produce T<sub>1</sub> seed, and T<sub>1</sub> plants containing a single *pat* and *aad-12* gene insertion were identified. DAS-81910-7 cotton was selected as the lead event based on molecular and phenotypic characteristics.

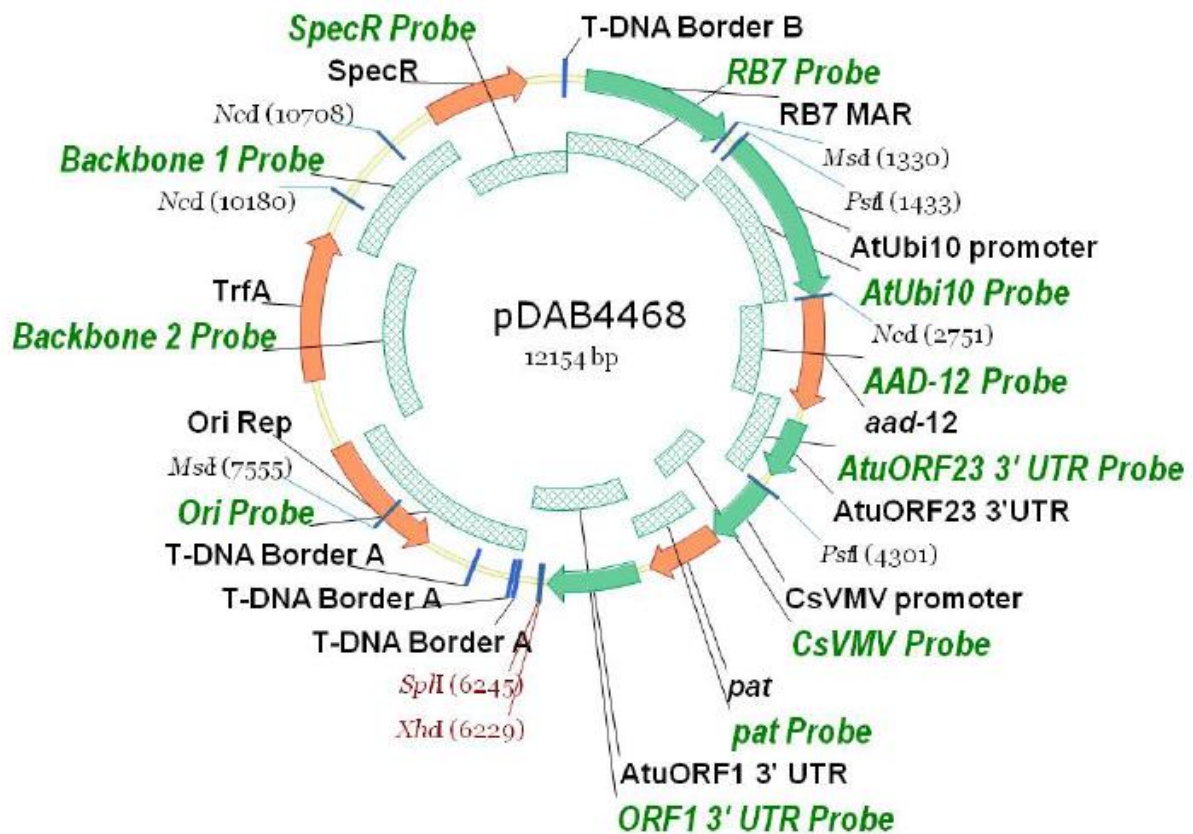


Figure 2: Vector map of plasmid pDAB4468 showing restriction enzyme sites and probe locations used in the molecular characterisation

### 3.2 Description of the introduced genes

A diagram of the T-DNA insert in plasmid pDAB4468 is given in Figure 2. Information on the genetic elements in the T-DNA insert is summarised in Table 1.

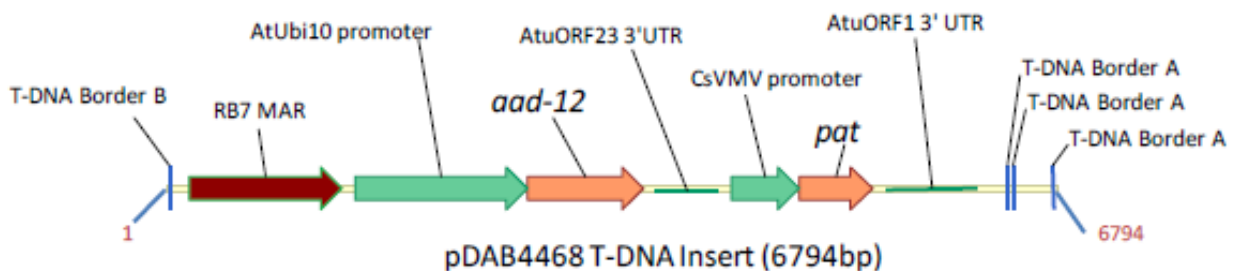


Figure 3: Representation of the genetic elements in the T-DNA insert of plasmid pDAB4468

Table 1: Description of the genetic elements contained in the T-DNA insert of pDAB4468

Genetic element	bp location on pDAB4468	Size (bp)	Source	Orient.	Description & Function	References
Border B	1 - 24	24	<i>Agrobacterium tumefaciens</i>		• Required for the transfer of the T-DNA into the plant cell	Barker <i>et al.</i> (1983)
<b>Matrix attachment region (MAR)</b>						
Intervening sequence	25 - 160	136			• Cloning sequence from Ti plasmid pTi15955	Barker <i>et al.</i> (1983)
RB7-MAR	161 - 1326	1166	<i>Nicotiana tabacum</i>	Clockwise	• Matrix attachment region • Increases expression and	Hall <i>et al.</i> (1991)

Genetic element	bp location on pDAB4468	Size (bp)	Source	Orient.	Description & Function	References
					reduces gene silencing	
<b>aad-12 cassette</b>						
Intervening sequence	1327 - 1421	95			• Cloning sequence from plasmid pENTR/D-TOPO	Invitrogen Cat. No. A10465
AtUbi10 promoter	1422 - 2743	1322	<i>Arabidopsis thaliana</i>	Clockwise	• Polyubiquitin 10 promoter, 5'UTR and intron • Drives constitutive expression of the <i>aad-12</i> gene	Norris <i>et al.</i> (1993)
Intervening sequence	2744 - 2751	8			• Cloning sequence	
<i>aad-12</i>	2752 - 3633	882	<i>Delftia acidovorans</i>	Clockwise	• Coding sequence of the aryloxyalkanoate dioxygenase gene • Optimised for expression in plants	Wright <i>et al.</i> (2007); Wright <i>et al.</i> (2010)
Intervening sequence	3634 - 3735	102			• Cloning sequence	
<i>AtuORF23</i> terminator	3736 - 4192	457	<i>Agrobacterium tumefaciens</i> pTi15955	Clockwise	• Transcriptional terminator and polyadenylation site of open reading frame 23	Barker <i>et al.</i> (1983)
Intervening sequence	4193 - 4306	114			• Cloning sequence from plasmid pENTR/D-TOPO	Invitrogen Cat. No. A10465
<b>pat cassette</b>						
CsVMV promoter	4307 - 4823	517	Cassava vein mosaic virus	Clockwise	• Drives constitutive expression of the <i>pat</i> gene	Verdaguer <i>et al.</i> (1996)
Intervening sequence	4824 - 4830	7			• Cloning sequence	
<i>pat</i>	4831 - 5382	552	<i>Streptomyces viridochromogenes</i> strain Tü494	Clockwise	• Providing glufosinate ammonium tolerance. • Optimized for plant codon usage.	Wohlleben <i>et al.</i> (1988)
Intervening sequence	5383 - 5484	102			Cloning Sequence from plasmid pCRI2.1	Invitrogen Cat. No. K205001
<i>AtuORF1</i> terminator	5485 - 6188	704	<i>Agrobacterium tumefaciens</i> pTi15955	Clockwise	• Transcriptional terminator and polyadenylation site of open reading frame 1	Barker <i>et al.</i> (1983)
Intervening sequence	6189 - 6416	228			• Cloning sequence from <i>Ti</i> plasmid C58	Zambryski <i>et al.</i> (1982); Wood <i>et al.</i> (2001)
<b>Border A</b>						
Border A	6417 - 6440	24	<i>Agrobacterium tumefaciens</i>		• Required for the transfer of the T-DNA into the plant cell	Barker <i>et al.</i> (1983)
Intervening sequence	6441-6459	19			• Cloning sequence from <i>Ti</i> plasmid C58	Zambryski <i>et al.</i> (1982); Wood <i>et al.</i> (2001)
Border A	6460-6483	24	<i>Agrobacterium tumefaciens</i>		• Border repeat	Zambryski (1988)
Intervening sequence	6484-6770	287			• Cloning sequence from <i>Ti</i> plasmid pTi15955	Barker <i>et al.</i> (1983)
Border A	6771-6794	24	<i>Agrobacterium tumefaciens</i>		• Border repeat	Zambryski (1988)

### 3.2.1 *aad-12* expression cassette

The *aad-12* gene of *D. acidovorans*, also referred to as *sdpA* (Schleinizt *et al.*, 2004; Wright *et al.*, 2007; Wright *et al.*, 2010) has low homology (approximately 37% sequence identity) with the *tda* gene first isolated from *Ralstonia eutropha* (Streber *et al.*, 1987) but found in phylogenetically diverse bacteria (Baelum *et al.*, 2008). The *tda* gene codes for an  $\alpha$ -ketoglutarate-dependent dioxygenase which converts chlorinated phenoxyalkanoate herbicides such as 2,4-D into a harmless phenol and glyoxylate.

Expression of the *aad-12* gene confers tolerance to both phenoxyalkanoate herbicides as well as to pyridyloxyacetic acids such as trichlopyr and fluroxypyr (Wright *et al.*, 2007). As well as occurring in *D. acidovorans*, *sdpA* (*aad-12*) genes have also been reported to occur in *Sphingomonas herbicidovorans* and *Rhodofera* sp. but show considerable sequence diversity (Paulin *et al.*, 2010).

The DNA sequence of the *aad-12* gene has been optimised for expression in plants and is approximately 80% identical to the DNA sequence of the native *aad-12* gene. The *aad-12* coding region in plasmid pDAB4468 is 882 bp in length and is driven by the constitutive polyubiquitin promoter from *Arabidopsis thaliana*. A matrix attachment region (MAR) from the root-specific *Rb7* gene (Hall, Jr. *et al.*, 1991; Verma *et al.*, 2005) of *Nicotiana tabacum* (tobacco) was included at the 5' end of cassette to potentially increase the consistency of *aad-12* expression (Abranches *et al.*, 2005). When positioned on the flanking ends of gene cassettes, some MARs have been shown to increase expression of transgenes and to reduce the incidence of gene silencing. At the 3' untranslated region of the coding region is a transcript termination and polyadenylation region from *Agrobacterium tumefaciens*.

### 3.2.2 pat expression cassette

The *pat* gene from *Streptomyces viridochromogenes* and the *bar* gene from *S. hygroscopicus* confer tolerance to herbicides containing glufosinate ammonium (phosphinothricin). Both genes code for polypeptides of 183 amino acids and share 87% homology at the nucleotide sequence level (Wehrmann *et al.*, 1996). Both genes have been widely used for genetic modification of food species.

The *pat* gene coding region (Strauch *et al.*, 1988; Wohlleben *et al.*, 1988) used in plasmid pDAB4468 is 552 bp in length and has been optimised for expression in plants. It is driven constitutively by a promoter region of the Cassava vein mosaic virus and terminated by a sequence of the 3' untranslated region of an open reading frame originating from plasmid pTi5955 of *Agrobacterium tumefaciens*.

## 3.3 Breeding process and analyses

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the molecular and genetic characteristics of cotton line 81910
- ensuring that the DAS-81910-7 event is incorporated into elite proprietary breeding line(s) for commercialisation..

The breeding pedigree for the various generations is given in Figure 4.

Following selection of T<sub>0</sub> plants (see Section 3.1) a series of self-fertilisation and seed bulking crosses proceeded up to generation T<sub>5</sub>. At the T<sub>3</sub> generation, plants were crossed with a number of elite lines to produce an F1 generation which was backcrossed to the appropriate parental elite cultivar and then self-fertilised for several generations.

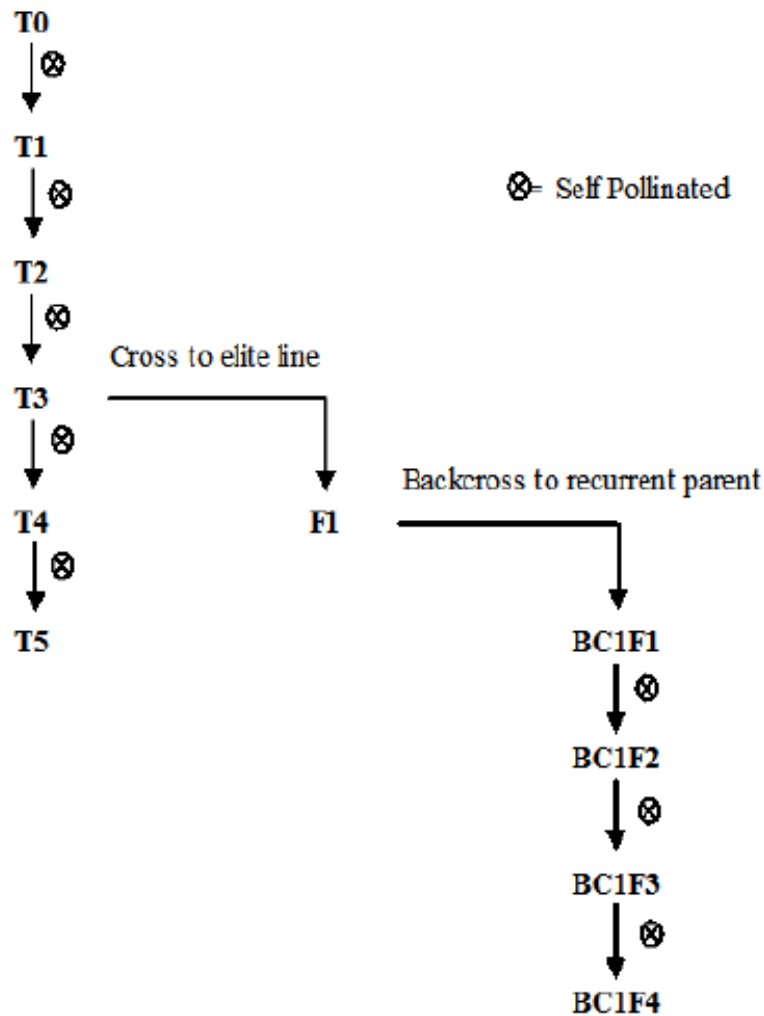


Figure 4: Breeding strategy for plants containing event DAS-81910-7

Table 2 indicates the generations that were used in the various studies characterising cotton line DAS-81910-7.

**Table 2: DAS-81910-7 generations used for various analyses**

Analysis	DAS-81910-7 Generation used	Control used
Molecular characterisation	T <sub>2</sub> , T <sub>3</sub> , T <sub>4</sub> , T <sub>5</sub> , BC <sub>1</sub> F <sub>2</sub>	'Coker 310'
Mendelian inheritance	BC <sub>1</sub> F <sub>2</sub> ,	
Genetic stability	T <sub>2</sub> , T <sub>3</sub> , T <sub>4</sub> , T <sub>5</sub> , BC <sub>1</sub> F <sub>2</sub>	
Protein characterisation and comparison of plant and microbial proteins	T <sub>3</sub>	'Coker 310'
Protein expression in plant parts	BC <sub>1</sub> F <sub>3</sub>	'Coker 310'
Compositional analyses	BC <sub>1</sub> F <sub>3</sub>	'Coker 310'

### 3.4 Characterisation of the genes in the plant

Analyses were undertaken in order to fully characterise, at the molecular level, the genetic modification in cotton line 81910.

These analyses focussed on the exact nature of the inserted genetic elements, and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure. A range of techniques including Southern blots, PCR and DNA sequence analysis were used for the characterisation.

### 3.4.1 Transgene copy number and insertion integrity

Total genomic DNA from pooled leaf tissue of each of 5 generations ( $T_2$ ,  $T_3$ ,  $T_4$ ,  $T_5$  and  $BC1F_2$ ) from cotton line 81910 (lateral flow strip-verified for presence of PAT) and from 'Coker 310' (negative control) was used for Southern blot analyses. A positive control (DNA from 'Coker 310' spiked with restriction enzyme-digested DNA from the pDAB4468 plasmid) was also included in the Southern blot analyses.

DNA from cotton line 81910, 'Coker 310' and the positive control was digested with several restriction enzymes (as indicated in Figure 2). The enzymes *NcoI*, *SphI* and *MscI* were chosen to determine the number of insertions, enzymes *PstI*, *PstI/XhoI* and *SphI* were used to characterise the structure of the genetic elements.

The resulting DNA fragments were separated and transferred to a membrane for sequential hybridisation with 11 different digoxigenin (DIG)-labelled probes (refer to Figure 2) that represent various sections of the T-DNA (7 functional regions) and vector backbone (4 regions) and were generated using a PCR DIG probe Synthesis Kit (Roche Diagnostics).

In some of the Southern analyses, the DNA from generation  $T_4$  consistently showed a band that ran slightly differently from the other generation samples. It is concluded that this is associated with the quality of the genomic DNA isolated from  $T_4$  and does not reflect a difference in actual digestion fragment size.

The Southern blot analyses verified that:

- Hybridisation bands were detected in the DNA from cotton line 81910 and the positive control at the expected size. No hybridisation bands were detected in the negative control. Therefore cotton line DAS-81910-7 contains a single insert.
- The single insert contains intact sequences of the *aad-12* and *pat* cassettes and of the RB7 MAR.
- There are no pDAB4468 backbone sequences present in the cotton line 81910 genome.

### 3.4.2 DNA sequence analysis

Analysis of PCR products and DNA sequencing allows the exact nature of the T-DNA insert in cotton line 81910 to be described. This allows confirmation of the organisation and sequence of each genetic element transferred into the plant, relative to the sequences in the T-DNA region of the transforming plasmid.

Genomic DNA was obtained from verified leaf tissue from the  $T_4$  generation of cotton line 81910. These samples were used to characterise the DNA sequence in the transgene insertion and its flanking border regions.

Four overlapping PCR fragments spanning the inserted sequences and border regions in event DAS-81910-7 were amplified, purified and then sub-cloned into a bacterial vector (pCR4-TOPO®). For each fragment, the DNA from vector colonies was sequenced individually and the sequences were aligned to obtain a consensus sequence.

Commercially available software (Sequencher®) was then used to assemble the consensus sequences to obtain a final sequence for the DAS-81910-7 insert. This sequence was then compared to the T-DNA sequence in pDAB4468.

A total of 8,834 base pairs (bp) of DAS-81910-7 sequences were obtained comprising 1,373 bp of 5' flanking border sequence, 1,071 bp of 3' flanking border sequence and 6,390 bp of insert (which includes two bp from Border B, 224 bp rather than 228 bp of the 3' intervening sequence and no DNA from Border A).

The sequences of the insert in event DAS-81910-7 confirmed that there is:

- A single intact copy of each of the aad-12 and pat cassettes.
- A single intact copy of the RB7 MAR element.

### **3.4.3 Novel open reading frame (ORF) analysis**

Bioinformatic assessment of any putative ORFs inherent to the inserted DNA or contiguous with the adjacent plant genomic DNA is used to identify whether any might encode a peptide with homology to known toxins or allergens, or otherwise indicate a need for further characterisation if translated. The bioinformatic analysis is entirely theoretical and does not inform on whether any of the ORFs are actually transcribed into RNA and translated into protein. Putative ORFs in all six reading frames are considered (that is, three forward reading frames and three in the reverse orientation).

The sequence of 8,834 bp obtained in the sequence analysis (Section 3.4.2) was analysed using an in-house Perl script to search for six-frame translations from stop codon to stop codon.

Within the insert, a total of 430 ORFs (greater than 8 amino acids) were identified. Eleven ORFs (greater than 8 amino acids), six at the 5' end and five at the 3' end, were identified spanning the junctions across the insert and its border regions. A discussion of the bioinformatic analysis of the novel ORFs is given in Section 4.1.

### **3.4.4 Analysis of the insertion site**

An analysis of the genomic region corresponding to the location of the DAS-81910-7 insert in the 'Coker 310' genome was undertaken to evaluate any DNA re-arrangements that might have occurred as a result of the transformation procedure.

Genomic DNA was obtained from verified leaf tissue from the T<sub>4</sub> generation of cotton line 81910 and from 'Coker 310'. Amplified PCR products generated from the 5' and 3' borders flanking the DAS-81910-7 insert were then compared with an amplicon (R3) obtained from 'Coker 310' that spanned the insertion site of DAS-81910-7. Comparison between R3 and the 5' and 3' borders flanking the DAS-81910-7 insert showed the following:

- nucleotides 1 – 631 from R3 are identical to nucleotides 743 – 1373 in the 5' border of the insert
- nucleotide 1373 is the 5' end genome-to-insert junction
- nucleotides 788 – 1861 from R3 are identical to the entire 3' border of the insert
- a 159 bp fragment of 'Coker 310' DNA has been deleted from the insertion site.



### 3.5 Stability of the genetic changes

Data demonstrating the stability of the introduced trait over a number of successive plant generations must be provided. Stability can be assessed both analytically and phenotypically. The molecular analyses include techniques such as Southern blots to probe specifically for the inserted DNA in seeds or other plant tissues from each generation. Phenotypic analysis refers to the observed expression of the introduced trait that is carried over to successive generations. Genetic stability can be quantified by a trait inheritance analysis (chemical, molecular and visual) of progeny to determine Mendelian heritability.

Phenotypic stability was assessed using greenhouse-grown plants of a segregating BC<sub>1</sub>F<sub>2</sub> generation of cotton line 81910 generated by crossing T<sub>3</sub> plants with an elite non-GM line. The F<sub>1</sub> plants were then self-pollinated to obtain the BC<sub>1</sub>F<sub>2</sub> generation (refer to Figure 4).

Leaves of 136 BC<sub>1</sub>F<sub>2</sub> cotton plants were analysed by lateral flow strip testing for expression of the AAD-12 protein. A Chi squared ( $X^2$ ) test for specified proportions was used to compare the observed segregation data to the hypothesised ratio of 3:1 (AAD-12 positive : AAD-12 negative). A total of 104 plants were positive for AAD-12 while 32 were negative. The  $X^2$  value of 0.1569 ( $P > 0.05$ ) indicated that the segregation ratio was consistent with the Mendelian inheritance pattern for a single independent locus.

The same plants were also tested by event-specific PCR for the presence/absence of the DAS-81910-7 insert. The results were entirely consistent with the AAD-12 protein results i.e. all plants testing positive for AAD-12 also tested positive for the insert, and all plants testing negative for AAD-12 also tested negative for the insert.

Genetic stability was assessed by the Southern blot analyses described in Section 3.4.2 which utilized DNA isolated from five generations of 81419. The hybridization patterns were identical across the five generations, thus indicating the integrity and stable inheritance of the insert.

### 3.6 Antibiotic resistance marker genes

No antibiotic resistance marker genes are present in cotton line 81910. Plasmid backbone analysis (refer to Section 3.4.1) shows that no plasmid backbone has been integrated into the soybean genome during transformation, i.e. the *specR* gene, which was used as a bacterial selectable marker gene, is not present in cotton line 81910.

### 3.7 Conclusion

Cotton line DAS-81910-7 contains two genes derived from bacteria that were introduced on a single expression cassette via *Agrobacterium*-mediated transformation. The *aad-12* gene from *Delftia acidovorans* encodes the AAD-12 protein, an  $\alpha$ -ketoglutarate-dependent dioxygenase that inactivates 2,4-D. The *pat* gene from *Streptomyces viridochromogenes* encodes phosphinothricin N-acetyltransferase (PAT), an enzyme conferring tolerance to herbicides containing glufosinate ammonium (phosphinothricin). Detailed molecular analyses of cotton line 81910 indicate that one complete copy of the two-gene expression cassette is present at a single insertion site. Plasmid backbone analysis shows no extraneous sequences, including antibiotic resistance genes derived from the plasmid were transferred to the cotton line 81910 genome.

The introduced genetic elements and the expression of new proteins in cotton line 81910 were shown by phenotypic analysis and molecular techniques to be stably inherited from one generation to the next across multiple generations.

The pattern of inheritance supports the conclusion that the herbicide-tolerance traits occur within a single locus in the cotton line 81910 genome and are inherited in accordance with Mendelian principles.

## 4. Characterisation of novel proteins

In considering the safety of newly expressed proteins, it is important to consider that ingestion of a large and diverse repertoire of proteins is part of a normal human diet. Almost all of the large numbers of proteins in foods are consumed without any adverse effects, although a small number have the potential to affect health, for example, because they are allergenic, or they have anti-nutritional properties (Delaney *et al.*, 2008). Proteins that are toxic in mammals are relatively rare; some examples include ricin from the castor oil plant and amatoxin oligopeptides from poisonous mushrooms.

As proteins perform a wide variety of biochemical functions in living organisms, their characteristics and possible effects are considered during the safety assessment of GM foods. This includes the potential of a newly expressed protein to be toxic, allergenic or exhibit anti-nutritional properties if present in the diet. To effectively identify any potential hazards requires knowledge of the characteristics of the newly expressed protein and its localisation and levels in plant tissues, particularly the food-producing parts of the plant. The evaluation includes a detailed understanding of the biochemical function and phenotypic effects of the newly expressed protein. It is also necessary to determine if any post-translational modifications are present, particularly any that were not evident in the source organism.

Two types of novel proteins were considered:

- those that may be potentially generated as a result of the creation of novel ORFs during the introduction of the T-DNA of plasmid pDAB4468 (see Section 3.4.3).
- those that were expected to be produced as a result of the expression of the introduced genes. Cotton line 81910 expresses three new proteins, AAD-12 and PAT.

### 4.1 Potential allergenicity/toxicity of novel ORFs created by the transformation procedure

#### Study submitted:

Guttikonda, S.K. (2013). Bioinformatics evaluation of the putative reading frames across the whole T-DNA insert and junctions in DAS-81910-7 cotton for potential protein allergenicity and toxicity. Study ID: 130619 Dow AgroSciences, LLC. Indianapolis, IN. (unpublished).

Eleven novel ORFs were identified in the flanking regions and 430 in the T-DNA insert itself (refer to Section 3.4.3). The amino acid sequences corresponding to these ORFs were analysed for potential allergenicity and toxicity using an *in silico* approach. These analyses are entirely theoretical since there is no reason to expect that any of the identified ORFs would, in fact, be expressed.

#### 4.1.1 Allergenicity assessment

The amino acid sequence of each identified ORF was compared with a peer-reviewed database containing 1,630 known and putative allergens, as well as coeliac-induction sequences residing in the FARRP (Food Allergy Research and Resource Program) dataset (Version 13 – February 2013) within AllergenOnline (University of Nebraska; <http://www.allergenonline.org/>).

The allergen search utilised the Fast Alignment Search Tool - All (FASTA) search algorithm, version 34, with Blocks Substitution Matrix50 (BLOSUM50) scoring matrix<sup>2</sup>. ORFs shorter than 29 amino acids were not evaluated since a minimum 35% identity requires at least a match of 29 amino acids over 80 amino acids. The 35% identity is a recommended criterion for indicating potential allergenicity (Codex, 2003; FAO/WHO, 2001).

A separate eight-amino-acid search comparing every possible peptide of eight contiguous amino acids in the query sequence with the sequences in the FARRP AllergenOnline database was also carried out.

Of the 11 ORFs identified in the flanking regions, five were less than 29 amino acids and of the 430 ORFs in the insert, 303 were less than 29 amino acids. For the remaining ORFs > 29 amino acids, in both the flanking regions and insert, no similarities with known allergens that exceeded the minimum 35% shared identity over a minimum of 80 amino acids were found. No matches of eight or more contiguous amino acids were found between any sequence and any entry in the FARRP AllergenOnline database.

#### **4.1.2 Toxicity assessment**

The sequences corresponding to the 11 identified ORFs in the flanking regions and 430 ORFs in the insert were compared with protein sequences present in a number of large public reference databases including Swissprot, PIR (Protein Information Resource), PRF (Protein Research Foundation) and PDB (Protein Data Bank). The similarity searches used the BLASTP (Basic Local Alignment Search Tool Protein) algorithm. BLASTP is now frequently applied for searching for similarities in protein sequences by performing local alignments of domains or short sequence similarities; this detects more similarities than would be found using the entire query sequence length. The search generates a parameter known as the *E* value (see eg Baxevanis, 2005). A statistically significant sequence similarity generally requires a match with an *E*-value of less than 0.01 (Pearson, 2000). Commonly, for protein-based searches, hits with *E*-values of  $10^{-3}$  or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis, 2005).

No significant similarity with an *E*-value of <1 was returned for the 11 ORFs in the flanking regions. The BLASTP search of the 430 ORFs in the T-DNA insert returned eight ORFs that showed alignments with an *E*-value <1.0. As expected, two of the alignments were with AAD-12 and PAT. None of the remaining six ORFs returned alignments with any known protein toxins.

#### **4.1.3 Conclusion**

It is concluded that, in the unlikely event any of the identified novel ORFs were expressed, there is no significant similarity between the encoded sequences and any known protein toxins or allergens.

## **4.2 The AAD-12 and PAT proteins**

Both the AAD-12 and PAT proteins have been considered to be safe by FSANZ in a number of different applications. For AAD-12, there have been two Applications – A1046 – soybean (FSANZ, 2011) and A1073 – soybean (FSANZ, 2013a).

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<sup>2</sup> The BLOSUM series of matrices tabulate the frequency with which different substitutions occur in conserved blocks of protein sequences and are effective in identifying distant relationships (Henikoff and Henikoff, 1992). The most commonly used BLOSUM matrices are BLOSUM50 (the default for use with the FASTA algorithm, with the matrix being built using sequences with no more than 50% similarity) and BLOSUM62 (the matrix used by BLAST and derived from blocks that are ≤ 62% identical).

The closely related AAD-1 protein was considered in A1042 – corn (FSANZ, 2010). The PAT protein, encoded by either the *pat* or *bar* genes (Wehrmann *et al.*, 1996), has now been considered in 18 FSANZ safety assessments (A372, A375, A380, A385, A386, A446, A481, A518, A533, A543, A589, A1028, A1040, A1046, A1073 A1080, A1081 and A1087) as well as being accepted in the literature as having neither toxicity nor allergenicity concerns (see e.g. Delaney *et al.*, 2008; Hérouet *et al.*, 2005).

A summary of these previous characterisations is provided in Table 3. For information, a reference is provided to the application in which the most recent detailed study or information was considered by FSANZ and is available on the FSANZ website. For the bioinformatic studies, which analyse sequence similarity to known protein toxins and allergens, where the Applicant provided searches using an updated (and hence larger) database, the results did not alter conclusions reached previously.

**Table 3: Summary of consideration of AAD-12 and PAT in previous FSANZ safety assessments**

Consideration	Sub-section	AAD-12 (link)	PAT (link)
Function and phenotypic effects		A1073 ( <a href="#">FSANZ, 2013a</a> )	A1087 ( <a href="#">FSANZ, 2013c</a> )
Potential toxicity	History of human consumption	A1073 ( <a href="#">FSANZ, 2013a</a> )	A1087 ( <a href="#">FSANZ, 2013c</a> )
	Amino acid sequence similarity to protein toxins	This application – using database updated February 2012-	A1087 ( <a href="#">FSANZ, 2013c</a> )
	<i>In vitro</i> digestibility	A1073 ( <a href="#">FSANZ, 2013a</a> )	A1080 ( <a href="#">FSANZ, 2013b</a> )
	Stability to heat	A1073 ( <a href="#">FSANZ, 2013a</a> )	A1080 ( <a href="#">FSANZ, 2013b</a> )
	Acute oral toxicity	A1046 ( <a href="#">FSANZ, 2011</a> )	A1080 ( <a href="#">FSANZ, 2013b</a> )
Potential allergenicity	Source of the protein	A1073 ( <a href="#">FSANZ, 2013a</a> )	A1087 ( <a href="#">FSANZ, 2013c</a> )
	Amino acid sequence similarity to allergens	This application – using database updated February 2013-	This application – using database updated February 2013

#### Studies submitted containing updated bioinformatic analyses

Mo J, 2012. Sequence similarity of AAD-12 protein to known toxins by bioinformatics analysis (update, February, 2012). Study ID 120142 Dow AgroSciences LLC. Indianapolis, IN.IN.  
 Song P, 2013a. Sequence similarity assessment of the AAD-12 protein to known allergens by bioinformatics analysis. Study ID 130068 Dow AgroSciences, LLC. Indianapolis, IN.  
 Song P, 2013b. Sequence similarity assessment of the PAT protein to known allergens by bioinformatics analysis. Study ID 130069 Dow AgroSciences, LLC. Indianapolis, IN.

#### 4.2.1 Characterisation of the plant-produced proteins

The AAD-12 protein expressed in cotton line 81910 is expected to comprise 293 amino acids and have an approximate molecular weight of 32 kDa. The Applicant claims the amino acid sequence is 99% homologous with the native AAD-12, differing only in that an alanine has been added at position 2 in order to facilitate cloning and optimise translation.

The PAT protein produced by cotton line 81910 should be identical to the native protein (Uniprot Accession No. Q57146). It is expected to comprise 183 amino acids and have an approximate molecular weight of 21 kDa.

**Studies submitted:**

Clement JM, Oman TJ, Juba AN, Singletary L, 2013. Characterization of the aryloxyalkanoate dioxygenase-12 (AAD-12) protein derived from transgenic cotton event DAS-81910-7. Study ID 110819 Dow AgroSciences LLC. Indianapolis, IN.

Embrey SK, 2011. Certificate of analysis of the test/reference/control substances: Aryloxyalkanoate dioxygenase-12 (AAD-12) – TSN030732. BIOT10-227507 Dow AgroSciences LLC. Indianapolis, IN.

Embrey SK, Juba AN, 2012. Certificate of analysis for phosphinothricin acetyl transferase (PAT, tsn303589) lyophilized protein standard Study ID: BIOT12-338281 Dow AgroSciences, LLC. Indianapolis, IN.

Karnoup A, Kuppannan K, 2008. Characterization of AAD-12: Batch TSN030732-002. ML-AL MD-2008-003833 The Dow Chemical Company. Midland, MI.

Oman TJ, Clement JM, Juba AN, Singletary LJ, 2013. Characterization of the phosphinothricin acetyltransferase (PAT) protein derived from transgenic cotton event DAS-81910-7. Study ID 120051 Dow AgroSciences. Indianapolis, IN

It is necessary to confirm that the proteins expressed in cotton line 81910 have the expected biochemical characteristics. Accordingly, the Applicant used a number of analytical techniques to characterise the AAD-12 and PAT proteins and compare them with previously characterised microbially-derived protein

The techniques used were:

- Sodium dodecyl polyacrylamide gel electrophoresis (SDS - PAGE)
- Western blot analysis
- Glycosylation analysis
- Peptide mass mapping
- Enzyme activity assay

Crude and partially immunopurified AAD-12 and PAT proteins were obtained from leaf tissue of verified cotton line 81910 (T<sub>3</sub> generation). Microbially-derived proteins had been produced and characterised from *Pseudomonas fluorescens*. As a control, a crude extract from leaf tissue of 'Coker 310' was used.

#### **4.2.1.1 Molecular weight and immunoreactivity of AAD-12 and PAT**

For the western blots, the nitrocellulose membrane was probed with:

- an AAD-12 polyclonal rabbit first antibody followed by goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase, or
- a PAT polyclonal rabbit first antibody followed by goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase

#### **AAD-12**

A Coomassie-stained SDS-PAGE gel of microbially-derived AAD-12 showed a single major band at approximately 32 kDa, which is the expected molecular weight. This band was immunoreactive in the western blot. Purified immunoreactive protein from cotton line 81910 also revealed a major band at 32 kDa on Coomassie-stained SDS-PAGE gel.

As expected, the crude plant extracts from both cotton line 81910 and 'Coker 310' showed numerous bands on a Coomassie-stained SDS-PAGE gel. In the western blot of cotton line 81910 crude extract, a band at 32 kDa showed immunoreactivity; no bands were detected in the western blot of 'Coker 310' crude extract.

## *PAT*

The gel of microbially-derived PAT showed a major band at approximately 20 kDa (the expected molecular weight) plus several faint higher molecular weight bands. The 20 kDa band was the only band that was immunoreactive in the western blot analysis.

As with the AAD-12 SDS gels, the crude plant extracts from both cotton line 81910 and 'Coker 310' showed numerous bands on a Coomassie-stained SDS-PAGE gel but in the western blot of cotton line 81910 crude extract, a band at 20 kDa showed immunoreactivity. No bands were detected in the western blot of 'Coker 310' crude extract.

### *Conclusion*

The Western blot analysis confirmed the plant- and microbial-derived proteins of each of AAD-12 and PAT were of equivalent molecular weight and immunoreactivity. It also indicated that the AAD-12 and PAT proteins expressed in cotton line 81910 are not fragmented, glycosylated or otherwise post-translationally modified since, for each protein, a single band was obtained with the expected mobility (molecular weight).

#### **4.2.1.2 Glycosylation status**

Many eukaryotic proteins are glycoproteins that have been post-translationally modified by the addition of carbohydrate moieties (glycans) covalently linked to the polypeptide backbone.

N-glycosylated proteins are glycosylated on an asparagine residue and commonly contain an asparagine-X-serine/threonine sequence (N-X-(P)-[S/T]), where X-(P) indicates any amino acid except proline (Orlando and Yang, 1998). Although rare, the sequence asparagine-X-Cysteine (N-X-C) can also be an N-glycosylation site (Miletich and Broze Jr., 1990). No N-glycosylation sites were predicted from the amino acid sequence of either AAD-12 or PAT.

Analysis of immunoaffinity-purified plant- and microbial-derived proteins was done using a commercial kit (GelCode Glycoprotein Staining Kit from ThermoScientific) following SDS-PAGE. The kit detects carbohydrates that may be covalently linked to the protein. A glycosylated protein (horseradish peroxidase) was applied to each gel as a positive control while the non-glycosylated protein, soybean trypsin inhibitor, was used as a negative control. A visible band was obtained for horseradish peroxidase while the soybean trypsin inhibitor and the AAD-12 and PAT immunopurified proteins from both plant and microbial sources gave no visible bands.

These results support the conclusion that neither microbially- nor DAS-81910-7-derived AAD-12 or PAT proteins are glycosylated and confirm the indication from the western blot analysis.

#### **4.2.1.3 Peptide mass mapping**

A protein identification made by peptide mass fingerprinting is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five peptide matches (Jensen *et al.*, 1997).

Purified AAD-12 and PAT proteins from cotton line 81910 were run on SDS-PAGE and the corresponding 32 kDa and 20 kDa protein bands, respectively, were excised. The AAD-12 and PAT bands were digested separately with each of trypsin and Asp-N (an endoproteinase that hydrolyzes peptide bonds on the N-terminal side of aspartic and cysteic acid residues) while the AAD-12 band was also digested with chymotrypsin.

The resulting peptide mixtures were then analysed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to determine the peptide mass fingerprint coverage. The sequences thus obtained were verified by tandem mass spectrometry (MS/MS). The masses of the detected peptides were searched *in silico* against those deduced from potential trypsin/Asp-N/chymotrypsin cleavage sites within the AAD-12 and PAT amino acid sequences, using Protein Analysis Worksheet freeware from Proteometrics LL.

For AAD-12, the combined MS sequence coverage from all digest analyses was approximately 88% of the 293 amino acids comprising the theoretical peptide sequence. For PAT, the combined sequence coverage was approximately 91% of the 183 amino acids.

The amino acid residues at the N- and C-termini of both plant-derived proteins were also determined and compared with microbially-derived protein. The analyses confirmed that the sequence of both the cotton line 81910-derived AAD-12 and PAT proteins matched that of the corresponding microbially-derived proteins. It also showed that the N-terminal Met of both proteins had been removed. Cleavage of the N-terminal Met by methionine aminopeptidase and amino-terminal acetylation is common, particularly in eukaryotes (Polevoda and Sherman, 2002; Polevoda and Sherman, 2003). For AAD-12, the analysis also showed the alanine at position 2 of the cotton line 81910-derived protein had been acetylated. The C-terminal sequences of each protein were as expected and were indistinguishable from the corresponding microbially-derived protein.

#### **4.2.1.4 Enzyme activity**

##### *AAD-12*

The enzymatic activity of immunopurified cotton line 81910 AAD-12 and microbially-produced AAD-12 was determined by a colorimetric assay (based on Fukumori and Hausinger, 1993) that measures released phenol derivative from a dichlorophenoxy compound (S-dichloroprop), varied over a concentration range from 0 – 125  $\mu$ M, in the presence of AAD-12. The expected hyperbolic curves were obtained for both the plant- and microbially-derived AAD-12 thus indicating their authenticity and functional equivalence.

##### *PAT*

The activity of plant- and microbially-derived PAT was measured using a spectrophotometric assay with minor modifications (De Block *et al.*, 1987; Mahan *et al.*, 2006). PAT was incubated with DL-glufosinate ranging from 25  $\mu$ M – 2 mM and the activity was quantified by measuring the liberation of the free CoA sulfhydryl group that forms concomitantly with transfer of the acetyl group from PAT to glufosinate. The expected hyperbolic curves were obtained for both the plant- and microbially-derived PAT thus indicating their authenticity and functional equivalence.

#### **4.2.1.5 Conclusion**

A range of characterisation studies confirmed the identity of AAD-12 and PAT derived from cotton line 81910 and also their equivalence with the corresponding protein produced in a bacterial expression system. The plant AAD-12 and PAT proteins have the expected molecular weight (32 kDa and 20 kDa respectively), immunoreactivity, lack of glycosylation, amino acid sequence and enzyme activity.

### 4.3 Novel protein expression in plant tissues

#### Study submitted:

Hill RC, (2013). Protein expression of a transformed cotton line containing aryloxyalkanoate dioxygenase (AAD-12) and phosphinothricin acetyltransferase (PAT) - event DAS-81910-7. Study ID 120040.02 Dow AgroSciences LLC. Indianapolis IN. (unpublished)

Expression of the AAD-12 and PAT proteins is expected in all plant tissues since the genes encoding them are driven by constitutive promoters (refer to Table 1).

Locations in the U.S.<sup>3</sup> representing regions where cotton is commercially grown, were planted with cotton line 81910 (generation BC1F3) and 'Coker 310' in 2012. This study used plant material from six of the same locations as described in Section 6 – Compositional Analysis. For the cotton line 81910 plants, there were two herbicide treatments – a) unsprayed and b) sprayed with 2,4-D (GF-2654) and glufosinate ammonium (Ignite 280 SL) at target rates of 1120 g ae/ha and 596 g ai/ha<sup>4</sup> respectively, applied at the 3-node and 6-node growth stages. Samples (4 pooled samples/site) for analysis of expression of AAD-12 and PAT were taken from each of a number of plant parts at specific growth stages (refer to Table 4).

The AAD-12 and PAT protein levels were determined by enzyme linked immunosorbent assay (ELISA) using commercial ELISA kits (from Acadia Bioscience, LLC for AAD-12 and from EnviroLogix Inc. for PAT). The limit of detection (LOD) and limit of quantitation (LOQ) were determined during method validation. For AAD-12, the LOD and LOQ were 0.5 µg/g and 1.0 µg/g respectively and for PAT were 0.025 µg/g and 0.06 µg/g respectively.

No AAD-12 proteins were detected in any samples taken from 'Coker 310' plants. Detectable levels of PAT were found in a total of seven 'Coker 310' samples (six of which were between the LOD and LOQ) spread over four separate stages and are likely due to sampling error and/or contamination.

Since the levels of AAD-12 and PAT in cotton line 81910 were similar for both unsprayed and sprayed plots, the data presented in Table 4 represent the results of combined analyses of the two treatments.

**Table 4: Average concentration (ug/g dw) over six locations and over two herbicide treatments of AAD-12 and PAT proteins in various plant parts from cotton line DAS-81910-7**

Sample source	Growth Stage*	Sample size	Average protein content in µg/g dry weight ± SD	
			AAD-12	PAT
Bolls	Peak bloom	10 – 14 bolls	17.17 ± 7.91	3.16 ± 1.11
Flower	Peak bloom	14 – 18 flowers	30.63 ± 8.36	5.30 ± 1.09
Leaf	4-leaf	10 – 14 leaves	71.17 ± 46.63	13.29 ± 4.76
Leaf	1 <sup>st</sup> white bloom	10 – 14 leaves	17.53 ± 8.60	8.18 ± 2.57
Leaf	1 <sup>st</sup> open boll	10 – 14 leaves	51.26 ± 19.63	9.14 ± 3.92

<sup>3</sup> The test sites were located in Alabama, Georgia, Louisiana, Missouri, North Carolina and Texas (refer to Section 6.2 for more detail)

<sup>4</sup> ae = acid equivalent; ai = active ingredient



Sample source	Growth Stage*	Sample size	Average protein content in µg/g dry weight ± SD	
			AAD-12	PAT
Pollen	Early bloom	0.2 – 0.5 ml	70.71 ± 19.58	0.11 ± 0.22
Root	maturity	1 – 2 plants	10.74 ± 5.27	1.63 ± 0.70
Seed	maturity	175 – 250 g	18.75 ± 4.81	3.85 ± 0.79
Squares	1 <sup>st</sup> white bloom	10 – 14 squares	38.33 ± 12.21	7.91 ± 2.39
Whole plant	maturity	1 – 2 plants	16.42 ± 12.18	0.97 ± 1.02

\*Information on cotton growth stages can be found in Ritchie *et al.* (2004)

Both AAD-12 and PAT were detected in all plant parts analysed. In general terms, it can be concluded that AAD-12 is present in highest concentration in 4-leaf stage leaves and pollen and lowest in the roots. For PAT, the highest level was in the 4-leaf leaves and the lowest in pollen.

Cottonseed would be the major source of human food produced from cotton line DAS-81910-7. It would be used to produce refined, bleached and deodorised oil, and to a smaller extent, linters which are highly processed and consist of nearly pure cellulose (>99%). The mean level of AAD-12 in cotton line 81910 is approximately 19 µg/g dw (0.0019%) and that of PAT is approximately 4 µg/g dw (0.0004%). As the mean percent dry weight of total protein in cotton line 81910 cottonseed is approximately 25% dw (see Table 5), the amount of AAD-12 protein in cotton line 81910 is calculated to be 0.008% of total protein and that of PAT to be 0.002%. In view of the low levels of AAD-12 and PAT in cottonseed, their presence in refined, bleached and deodorised (RBD) oil will be difficult to detect.

## 5. Herbicide metabolites

As part of the safety assessment it is important to establish whether the expression of a novel protein(s) is likely to result in the accumulation of any novel metabolites. If such substances are found to occur as a result of the genetic modification, then it is important to determine their potential toxicity.

The herbicide residues resulting from the application of 2,4-D and glufosinate ammonium to lines carrying the *aad-12* and *pat* genes have been assessed in previous applications (see Application A1046 (FSANZ, 2011) and Application A1073 (FSANZ, 2013a). There are no concerns that the spraying of cotton line 81910 with 2,4-D or glufosinate ammonium would result in the production of any novel metabolites that have not been previously considered.

## 6. Compositional analysis

The purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where the genetic modification has resulted in a deliberate change to one or more nutrients in the food. In this case, cotton line 81910 is herbicide tolerant and there was no intention to alter the nutrient composition of food derived from this plant line.

The focus of the compositional analysis is on those constituents most relevant to the safety of the food, or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients relevant for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors/anti-nutrients) or quantitatively more minor constituents (minerals, vitamins). Key toxicants are those that have a level of toxicity and occur in amounts that may be significant to health (eg solanine in potatoes).

## 6.1 Key components

Fuzzy cottonseed is the most usual source of food products derived from cotton. Cottonseed oil is the primary cotton product used for human consumption. For cotton, the key components that should be considered in the comparison include proximates (cottonseed only), fatty acids, tocopherol, gossypol and the cyclopropenoid fatty acids - malvalic, sterculic and dihydrosterculic acids (OECD, 2004). The Applicant also undertook analyses for amino acid and minerals, which are of greater relevance for animal feed.

## 6.2 Study design and conduct

### Study submitted:

Fast BJ, Johnson TY, (2013). Nutrient composition of a cotton cultivar containing aryloxyalkanoate dioxygenase-12 (AAD-12) and phosphinothricin acetyltransferase (PAT): Event DAS-81910-7. Study ID 120040.01 Dow AgroSciences LLC. Indianapolis IN. (unpublished).

The test (verified cotton line 81910 seed of BC1F3 lineage), and control (verified 'Coker 310') lines were grown under similar conditions at eight field sites across North America<sup>5</sup> during 2012 (with six of these sites also being used for the novel protein characterisation analysis described in Section 4.3). The sites were representative of locations where cotton is commercially grown. 'Coker 310' is the original transformed line and therefore represents the near-isogenic control line for the purposes of the comparative analyses (see Section 2.1). Six different commercial, non-GM cotton lines were also grown under the same conditions in order to generate a reference range for each analyte. The reference varieties were randomized across sites with three reference varieties at each site and each reference variety present at four sites.

Standard agronomic practices to reduce injury from insect, weed and disease were carried out at all sites. For the cotton line 81910 plants, there were two additional herbicide treatments – a) unsprayed and b) sprayed with 2,4-D (GF-2654) and glufosinate ammonium (Ignite 280 SL) at target rates of 1120 g ae/ha and 596 g ai/ha<sup>6</sup> respectively, applied at the 3-node and 6-node growth stages. As non-GM cotton is very susceptible to 2,4-D, even from drift from adjacent spraying, the cotton line 81910 plants receiving herbicide treatment were spatially separated from the control and reference variety entries. The study was therefore divided into two sub-experiments:

- sub-experiment 1 contained 'Coker 310', cotton line 81910 and reference varieties that did not receive 2,4-D applications
- sub-experiment 2 contained cotton line 81910 sprayed with 2,4-D + glufosinate and an additional entry of unsprayed plants of cotton line 81910.

<sup>5</sup> The eight sites were: Tallassee AL; Sycamore, GA; Washington, LA; Fisk, MO; Greenville, MS; Mebane, NC; Groom and East Bernard, TX

<sup>6</sup> ae = acid equivalent; ai = active ingredient

Plants were grown in a randomised complete block design within each sub-experiment, and the two sub-experiments were separated by 30 m to minimise possible drift injury to the unsprayed plants in sub-experiment 1.

Since the 'Coker 310' control and cotton line 81910 sprayed with 2,4-D + glufosinate entries were in separate sub-experiments, there was no direct comparison between the two. However, the following logic and comparisons allowed an indirect comparison to be made:

- an unsprayed cotton line 81910 treatment was included in both sub-experiment 1 and sub-experiment 2.
- equivalence between unsprayed 'Coker 310' and sprayed cotton line 81910 can be inferred by first comparing sprayed cotton line 81910 with unsprayed cotton line 81910 in sub-experiment 2.
- if no statistically significant difference in this above comparison exists then this indicates the application of herbicide has no significant effect on composition and therefore that sprayed cotton line 81910 is equivalent to unsprayed cotton line 81910.
- providing the above non-significance can be demonstrated, it is legitimate to use unsprayed cotton line 81910 (rather than sprayed cotton line 81910) for the comparison with the non-GM control (unsprayed 'Coker 310') in sub-experiment 1.
- if this comparison also produces no statistically significant difference, it can be inferred that both unsprayed cotton line 81910 and sprayed cotton line 81910 are equivalent to unsprayed 'Coker 310'.

Cottonseed samples from all plots were harvested at maturity and acid delinted before despatch to analytical laboratories under full identity labelling. In total, 73 analytes were analysed. Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

Fourteen of the 73 analytes included in the study were excluded from the combined site statistical analysis because more than 50% of the results for those analytes were less than the LOQ. For the remaining 59 analytes, data were transformed into Statistical Analysis Software<sup>7</sup> (SAS) data sets. A mean value was generated and used for each analyte comparison, and standard error and minimum and maximum values were also calculated for each analyte. A mixed model analysis of variance was used by the Applicant to test results across all field sites (combined site analysis) and within each field site (individual site analysis). The significance of an overall treatment effect was estimated using an F-test, while paired contrasts were made using t-tests. Probability values from the paired contrasts were adjusted using False Discovery Rate (FDR) procedures to improve discrimination of true differences (Benjamini and Hochberg, 1995). In assessing the significance of any difference between the mean analyte value for cotton line 81910 and 'Coker 310' an FDR-adjusted P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

The statistical results for the treatments were compared to:

- The reference range (min – max) compiled from the results of the six non-GM reference lines, in order to assess whether any differences were likely to be biologically meaningful.

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<sup>7</sup> SAS website - <http://www.sas.com/technologies/analytics/statistics/stat/index.html>

- A combined literature range for each analyte, compiled from published literature<sup>8</sup>. Any mean value for a cotton line 81910 analyte that fell within the combined literature range was considered to be within the normal variability of commercial cotton cultivars even if the mean value was statistically different from the 'Coker 310' control. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within cotton. Therefore, even if means fall outside the published range, this is not necessarily a concern.

### 6.2.1 Equivalence of sprayed and unsprayed cotton line DAS-81910-7

The comparison between sprayed cotton line 81910 and unsprayed cotton line 81910 in sub-experiment 2 (data not shown) indicated that for 21 of the analytes a significant difference was obtained at one of the eight sites in the individual site analysis and that for two of the analytes – aspartate and behenic acid - there was a significant difference at more than one site in the individual site analyses. However, there was no significant difference for any of the 59 analytes included in the combined site analysis. Therefore, it is concluded that cotton line DAS-81910-7 sprayed with 2,4-D + glufosinate is compositionally equivalent to unsprayed cotton line DAS-81910-7. Thus, in sub-experiment 1, where there is no significant difference between unsprayed cotton line DAS-81910-7 and 'Coker 310' this can be extended to mean that there is also no difference between sprayed cotton line DAS-81910-7 and 'Coker 310'.

The results summarised in Tables 5 – 10 are mainly for sub-experiment 1 but also contain the mean values obtained for sprayed cotton line DAS-81910-7 from sub-experiment 2. The probability (P) values pertain only to sub-experiment 1.

## 6.3 Seed composition

### 6.3.1 Proximates and fibre

Results for levels of proximate and fibre are shown in Table 5. Statistically significant differences between 'Coker 310' and cotton line 81910 were noted in the over-all analysis for protein, fat and ash but in all cases the means of cotton line 81910 were within both the range reported in the literature and the reference range. The FDR-adjusted P values for the pairwise analysis of these three analytes were not significant.

**Table 5: Mean percentage ( $\pm$  SD) of proximates and fibre in seed from 'Coker 310' and cotton line DAS-81910-7**

Analyte	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
Protein (%dw)	26.4 $\pm$ 0.9	25.4 $\pm$ 0.9	0.008	NS	21.5 – 32.3	24.7 $\pm$ 1.0	11.7 – 34.2
Fat (%dw)	21.7 $\pm$ 0.7	21.3 $\pm$ 0.7	NS	NS	15.8 – 27.9	20.5 $\pm$ 0.9	11.8 – 36.3
Ash (%dw)	4.29 $\pm$ 0.11	4.17 $\pm$ 0.11	0.047	NS	3.53 – 5.21	4.28 $\pm$ 0.13	3.2 – 5.34
Moisture (%fw)	8.2 $\pm$ 0.2	8.0 $\pm$ 0.2	NS	NS	6.37 – 10.2	8.2 $\pm$ 0.2	2.2 – 15.9
Carbohydrate (%dw) <sup>2</sup>	47.5 $\pm$ 0.9	49.1 $\pm$ 0.9	0.007	NS	42.3 – 54.2	50.5 $\pm$ 1.1	36.4 – 74.4

<sup>8</sup> Published literature for cotton included Kohel *et al.* (1985), Belyea *et al.* (1989), Berberich *et al.* (1996), Nida *et al.* (1996), Hamilton *et al.* (2004), OECD (2004), Bertrand *et al.* (2005), ILSI (2010) and Codex (2001)

Analyte	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
Total dietary fibre (%dw)	44.8 ± 1.0	45.7 ± 1.0	NS	NS	37.6 – 51.3	47.2 ± 1.1	33.7 – 47.5
Crude fibre (%dw)	18.1 ± 0.3	17.9 ± 0.3	NS	NS	15.1 – 23.5	17.8 ± 0.3	13.4 – 23.1
ADF <sup>3</sup> (%dw)	25.9 ± 0.5	25.3 ± 0.5	NS	NS	20.4 – 29.4	25.7 ± 0.5	19.7 – 66.9
NDF <sup>4</sup> (%dw)	34.0 ± 0.6	33.8 ± 0.6	NS	NS	27.2 – 38.2	34.8 ± 0.6	25.5 – 71.4

<sup>1</sup> Mauve shading represents cotton line 81910 means where an overall treatment effect showed a significantly lower value than for the 'Coker 310' mean, while orange shading represents cotton line 81910 means that were significantly higher than 'Coker 310'

<sup>2</sup> Carbohydrate calculated as 100% - (protein %dw + fat %dw + ash %dw)

<sup>3</sup> ADF = acid detergent fibre

<sup>4</sup> NDF = neutral detergent fibre

### 6.3.2 Fatty Acids

The major food from cotton plants is cottonseed oil and therefore the fatty acid constituents of cottonseed are highly relevant for safety assessment. The levels of 22 fatty acids (C8 – C22) were measured in seed from cotton line 81910 and 'Coker 310'. Of these, 13 were below the LOQ in more than 50% of samples and were therefore not statistically analysed: caprylic (8:0), capric (10:0), lauric (12:0), myristoleic (14:1), pentadecanoic (15:0), pentadecenoic (15:1), heptadecanoic (17:0), heptadecenoic (17:1),  $\gamma$ -linolenic (18:3), eicosenoic (20:1), eicosadienoic (20:2), eicosatrienoic (20:3), and arachidonic (20:4).

Results for the remaining nine fatty acids are given in Table 6 and can be summarised as follows:

- There was no significant difference between seed of 'Coker 310' and cotton line 81910 in terms of the levels of stearic, linolenic, arachidic and behenic acids.
- For myristic, palmitoleic and oleic acids, the mean levels in cotton line 81910 were significantly lower than that of 'Coker 310' both at the overall treatment level and for the FDR-adjusted P-value for a pairwise t-test comparison. However, the cotton line 81910 means fell within both the reference range and the literature range, except for the palmitoleic acid level in sprayed cotton line 81910 in sub-experiment 2, where the mean was marginally outside (lower than) the combined literature range. This does not raise any safety concerns.
- For linoleic acid, the level in cotton line 81910 was significantly higher both at the overall treatment level and for the FDR-adjusted P-value comparison than that of 'Coker 310'. The cotton line 81910 mean fell within the reference range but was marginally outside (higher than) the combined literature range. This does not raise any safety concerns.
- Palmitic acid in cotton line 81910 seed was significantly lower in the overall analysis but the FDR-adjusted P-value for the pairwise comparison was not significant and the means for both sprayed and unsprayed cotton line 81910 were within both the reference range and literature range.

**Table 6: Mean percentage composition ( $\pm$  SD), relative to total fat, of major fatty acids in seed from 'Coker 310' and cotton line DAS-81910-7**

Fatty acid	Sub-experiment 1					Sub-experiment 2	Combined literature range (% total)
	'Coker 310' (% total)	Unsprayed 81910-7 (% total) <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range (% total)	Sprayed 81910-7 (% total)	
Myristic (14:0)	0.72 $\pm$ 0.04	0.65 $\pm$ 0.04	< 0.001	0.002	0.43 – 1.04	0.64 $\pm$ 0.04	0.45 – 2.40
Palmitic (16:0)	22.55 $\pm$ 0.58	22.26 $\pm$ 0.58	0.027	NS	18.76 – 26.07	22.16 $\pm$ 0.66	15.11 – 28.10
Palmitoleic (16:1)	0.49 $\pm$ 0.02	0.46 $\pm$ 0.02	< 0.001	0.003	0.37 – 0.63	0.45 $\pm$ 0.02	0.46 – 1.19
Stearic (18:0)	4.29 $\pm$ 0.11	4.17 $\pm$ 0.11	NS	NS	3.53 – 5.21	4.28 $\pm$ 0.13	3.2 – 5.34
Oleic (18:1)	14.84 $\pm$ 0.35	13.95 $\pm$ 0.35	< 0.001	0.001	12.93 – 17.09	13.8 $\pm$ 0.34	12.8 – 25.3
Linoleic (18:2)	58.5 $\pm$ 0.8	59.7 $\pm$ 0.8	< 0.001	0.003	52.36 – 63.9	60.0 $\pm$ 0.9	46.00 – 59.4
Linolenic (18:3)	0.20 $\pm$ 0.008	0.21 $\pm$ 0.008	NS	NS	0.14 – 0.25	0.21 $\pm$ 0.009	0.11 – 0.35
Arachidic (20:0)	0.25 $\pm$ 0.01	0.25 $\pm$ 0.01	NS	NS	0.185 – 0.342	0.25 $\pm$ 0.01	0.186 – 0.414
Behenic (22:0)	0.137 $\pm$ 0.006	0.134 $\pm$ 0.006	NS	NS	0.103 – 0.174	0.135 $\pm$ 0.007	0.05 – 0.295

<sup>1</sup> Mauve shading represents cotton line 81910 means where an overall treatment effect showed a significantly lower value than for the 'Coker 310' mean, while orange shading represents cotton line 81910 means that were significantly higher than 'Coker 310'

### 6.3.3 Amino Acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine. Results of the analysis are given in Table 7.

The only significant difference (overall) between cotton line 81910 and 'Coker 310' was in the mean level of arginine but the FDR-adjusted P value for a pairwise t-test comparison of the same analyte was not significant. The mean arginine level in cotton line 81910 was within both the reference range and combined literature range.

**Table 7: Mean percentage composition ( $\pm$  SD), relative to total amino acids, of amino acids in seed from 'Coker 310' and cotton line DAS-81910-7**

Amino acid	Sub-experiment 1					Sub-experiment 2	Combined literature range (% total)
	'Coker 310' (% total)	Unsprayed 81910-7 (% total) <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range (% total)	Sprayed 81910-7 (% total)	
Alanine	4.44 $\pm$ 0.03	4.45 $\pm$ 0.03	NS	NS	4.18 – 4.65	4.49 $\pm$ 0.05	4.08 – 5.30
Arginine	12.64 $\pm$ 0.16	12.49 $\pm$ 0.16	0.02	NS	11.57 – 13.67	12.39 $\pm$ 0.20	10.83 – 15.18
Aspartate	10.09 $\pm$ 0.15	10.25 $\pm$ 0.15	NS	NS	9.59 – 11.42	10.56 $\pm$ 0.22	9.00 – 12.37
Cystine	1.78 $\pm$ 0.04	1.83 $\pm$ 0.04	NS	NS	1.59 – 2.34	1.78 $\pm$ 0.03	1.53 – 2.35
Glutamate	20.11 $\pm$ 0.13	20.07 $\pm$ 0.13	NS	NS	19.44 – 21.23	19.83 $\pm$ 0.20	20.24 – 22.90
Glycine	4.42 $\pm$ 0.04	4.41 $\pm$ 0.04	NS	NS	4.10 – 4.59	4.44 $\pm$ 0.05	4.29 – 5.72

Amino acid	Sub-experiment 1					Sub-experiment 2	Combined literature range (% total)
	'Coker 310' (% total)	Unsprayed 81910-7 (% total) <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range (% total)	Sprayed 81910-7 (% total)	
Histidine	2.84 ± 0.016	2.86 ± 0.016	NS	NS	2.65 – 3.07	2.83 ± 0.019	2.91 – 3.88
Isoleucine	3.62 ± 0.02	6.31 ± 0.02	NS	NS	3.18 – 3.81	3.62 ± 0.03	3.10 – 4.46
Leucine	6.30 ± 0.02	6.31 ± 0.02	NS	NS	6.04 – 6.54	6.32 ± 0.04	6.03 – 8.114.69 ± 0.06
Lysine	4.69 ± 0.06	4.73 ± 0.06	NS	NS	4.27 – 5.03	4.74 ± 0.07	4.62 – 6.60
Methionine	1.64 ± 0.023	1.62 ± 0.023	NS	NS	1.34 – 1.78	1.645 ± 0.033	1.27 – 2.28
Phenylalanine	5.70 ± 0.06	5.68 ± 0.06	NS	NS	5.44 – 6.02	5.63 ± 0.06	5.44 – 7.23
Proline	4.04 ± 0.01	4.04 ± 0.01	NS	NS	3.78 – 4.19	4.05 ± 0.02	3.81 – 5.30
Serine	4.63 ± 0.04	4.63 ± 0.04	NS	NS	4.26 – 5.05	4.63 ± 0.03	4.15 – 5.87
Threonine	3.54 ± 0.03	3.54 ± 0.03	NS	NS	3.18 – 3.75	3.58 ± 0.04	2.67 – 4.26
Tryptophan	1.43 ± 0.019	1.42 ± 0.019	NS	NS	1.29 – 1.67	1.42 ± 0.018	0.91 – 1.40
Tyrosine	3.33 ± 0.015	3.31 ± 0.015	NS	NS	3.19 – 3.46	3.32 ± 0.02	2.63 – 3.46
Valine	4.75 ± 0.02	4.74 ± 0.02	NS	NS	4.36 – 5.02	4.74 ± 0.04	4.49 – 6.24

<sup>1</sup>Mauve shading represents cotton line 81910 means where an overall treatment effect showed a significantly lower value than for the 'Coker 310' mean

#### 6.3.4 Minerals

Levels of 12 minerals were measured (Table 8). Of these, copper and manganese levels in cotton line 81910 seed were significantly lower than the levels in 'Coker 310', when an overall treatment effect was considered, but only manganese was significantly lower when an FDR-adjusted P value for a pairwise t-test comparison was considered. Irrespective of this, the mean levels of copper and manganese in cotton line 81910 seed were within both the reference and literature ranges.

**Table 8: Mean values (± SD) for mineral levels in seed from 'Coker 310' and cotton line DAS-81910-7**

Mineral	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
Calcium (mg/100 g dw)	124 ± 7	133 ± 7	0.011	NS	78.9 - 204	136 ± 7	100 - 330
Copper (mg/100 g dw)	0.90 ± 0.07	0.86 ± 0.07	0.02	NS	0.46 – 1.44	0.86 ± 0.08	0.313 – 2.45
Iron (mg/100 g dw)	4.20 ± 0.24	4.25 ± 0.24	NS	NS	3.43 – 6.45	4.30 ± 0.21	3.67 – 31.83

Mineral	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
Magnesium (mg/100 g dw)	387 ± 18	384 ± 18	NS	NS	285 - 470	388 ± 19	340 – 493.12
Manganese (mg/100 g dw)	1.59 ± 0.10	1.35 ± 0.10	0.002	0.02	0.983 – 2.28	1.31 ± 0.08	1.069 – 2.216
Molybdenum (mg/100 g dw)	0.039 ± 0.012	0.036 ± 0.012	NS	NS	0.003 – 0.122	0.034 ± 0.01	NR <sup>2</sup>
Phosphorus (mg/100 g dw)	652 ± 42	633 ± 42	NS	NS	460 - 901	649 ± 45	482.5 – 991.5
Potassium (mg/100 g dw)	1078 ± 23	1055 ± 23	NS	NS	938 - 1290	1070 ± 23	960 – 1448.3
Selenium (ppb dw)	110 ± 30	122 ± 30	NS	NS	<LOQ - 676	180 ± 95	NR
Sodium (mg/100 g dw)	123 ± 8	111 ± 8	NS	NS	73.9 - 192	136 ± 9	5.4 - 740
Sulfur (mg/100 g dw)	495 ± 67	535 ± 67	NS	NS	331 - 847	499 ± 74	NR
Zinc (mg/100 g dw)	3.36 ± 0.13	3.47 ± 0.13	NS	NS	2.77 – 4.26	3.48 ± 0.21	2.70 – 5.95

<sup>1</sup>Maue shading represents cotton line 81910 means where an overall treatment effect showed a significantly lower value than for the 'Coker 310' mean

<sup>2</sup>NR = not reported

### 6.3.5 Vitamins

Levels of seven vitamins were measured. Of these, the level of vitamin A ( $\beta$ -carotene) was below the LOQ in more than 50% of samples and was therefore not statistically analysed.

Overall, there were no significant differences between the cotton line 81910 means and the 'Coker 310' means for any of the analytes (Table 9). All means were within the reference range and, in the case of  $\alpha$ -tocopherol, also the combined literature range.

**Table 9: Mean values ( $\pm$  SD) for vitamin levels in seed from 'Coker 310' and cotton line DAS-81910-7**

Vitamin	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
$\alpha$ -tocopherol (mg/kg dw)	90 ± 11	88 ± 11	NS	NS	31.1 - 151	89 ± 10	70.82 – 197.2
Vitamin B1 (mg/kg dw)	10.3 ± 0.4	10.3 ± 0.4	NS	NS	5.54 – 14.7	10.4 ± 0.5	NR <sup>1</sup>
Vitamin B2 (mg/kg dw)	6.2 ± 0.5	6.3 ± 0.5	NS	NS	3.44 – 9.52	6.6 ± 0.5	NR
Vitamin B3 (mg/kg dw)	27.8 ± 1.2	28.5 ± 1.2	NS	NS	20.4 – 36.8	28.1 ± 1.1	NR
Vitamin B6 (mg/kg dw)	3.83 ± 0.09	3.72 ± 0.09	NS	NS	2.84 – 5.12	3.77 ± 0.13	NR



Vitamin	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
Vitamin B9 (mg/kg dw)	1.66 ± 0.77	1.67 ± 0.07	NS	NS	1.10 – 2.40	1.64 ± 0.09	NR

<sup>1</sup>NR = not reported

### 6.3.6 Anti-nutrients

Cottonseed samples from cotton line 81910 and 'Coker 310' were analysed for five anti-nutrient compounds characteristic of cotton. Results are given in Table 10 and can be summarised as follows:

- There was no significant difference between seed of 'Coker 310' and cotton line 81910 in terms of the levels of dihydrosterculic acid and sterculic acid.
- For malvalic acid the mean level in cotton line 81910 was significantly higher than that of 'Coker 310' both at the overall treatment level and for the FDR-adjusted P-value for a pairwise t-test comparison. However, the cotton line 81910 mean fell within both the reference range and the literature range.
- For free gossypol, the level in cotton line 81910 was significantly lower both at the overall treatment level and for the FDR-adjusted P-value comparison than that of 'Coker 310'. However, the cotton line 81910 mean fell within both the reference range and the literature range.
- The level of total gossypol in cotton line 81910 seed was significantly lower in the overall analysis but the FDR-adjusted P-value for the pairwise comparison was not significant and the mean was within both the reference range and literature range.

**Table 10: Mean values (± SD) for anti-nutrient levels in seed from 'Coker 310' and cotton line DAS-81910-7**

Anti-nutrient	Sub-experiment 1					Sub-experiment 2	Combined literature range
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	P (overall effect)	FDR-adjust. P (pairwise compar.)	Reference range	Sprayed 81910-7	
Manganese (mg/100 g dw)	1.59 ± 0.10	1.35 ± 0.10	0.002	0.02	0.983 – 2.28	1.31 ± 0.08	1.069 – 2.216
Malvalic acid (% total FA)	0.524 ± 0.023	0.577 ± 0.023	0.003	0.029	0.402 – 0.854	0.612 ± 0.026	0.17 – 0.759
Sterculic acid (% total FA)	0.275 ± 0.011	0.297 ± 0.011	NS	NS	0.196 – 0.44	0.301 ± 0.011	0.13 – 0.56
Free gossypol (% dw)	0.96 ± 0.06	0.83 ± 0.06	0.01	NS	0.492 – 1.28	0.81 ± 0.06	0.454 – 1.399
Total gossypol (% dw)	1.08 ± 0.05	0.95 ± 0.05	0.001	0.01	0.551 – 1.41	0.93 ± 0.05	0.547 – 1.522

<sup>1</sup> Mauve shading represents cotton line 81910 means where an overall treatment effect showed a significantly lower value than for the 'Coker 310' mean, while orange shading represents cotton line 81910 means that were significantly higher than 'Coker 310'

## 6.4 Conclusion from compositional analysis

Mean values for a total of 59 analytes are presented in Tables 5 – 10. Having established, from sub-experiment 2 that there is no compositional difference between unsprayed cotton line 81910 and cotton line 81910 sprayed with 2,4-D and glufosinate, the trends in the statistical outcomes relating to unsprayed cotton line 81910 in sub-experiment 1, as shown in these tables, can be applied to both sprayed and unsprayed cotton line 81910.

A summary of the seven analytes in which an FDR-adjusted P value for a pairwise t-test comparison showed a significant difference between cotton line 81910 and 'Coker 310' is provided in Table 11.

**Table 11: Summary of analyte means found in seed of cotton line DAS-81910-7 that are significantly (adj. P < 0.05) different from those found in seed of the control line 'Coker 310'**

Analyte	Sub-experiment 1			Sub-experiment 2	81910-7 within Combined literature range?
	'Coker 310'	Unsprayed 81910-7 <sup>1</sup>	81910-7 within reference range?	Sprayed 81910-7	
Myristic (14:0)	0.72 ± 0.04	0.65 ± 0.04	yes	0.64 ± 0.04	yes
Palmitoleic (16:1)	0.49 ± 0.02	0.46 ± 0.02	yes	0.45 ± 0.02	yes
Oleic (18:1)	14.84 ± 0.35	13.95 ± 0.35	yes	13.8 ± 0.34	yes
Linoleic (18:2)	58.5 ± 0.8	59.7 ± 0.8	yes	60.0 ± 0.9	Sprayed and unsprayed 81910 marginally higher
Manganese (mg/100 g dw)	1.59 ± 0.10	1.35 ± 0.10	yes	1.31 ± 0.08	yes
Malvalic acid (% total FA)	0.524 ± 0.023	0.577 ± 0.023	yes	0.612 ± 0.026	yes
Total gossypol (% dw)	1.08 ± 0.05	0.95 ± 0.05	yes	0.93 ± 0.05	yes

<sup>1</sup> Mauve shading represents cotton line 81910 means where an FDR adjusted P value showed a significantly lower value than for the 'Coker 310' mean, while orange shading represents cotton line 81910 means that were significantly higher than 'Coker 310'

For each of the analytes in Table 11, the differences were small in magnitude, and were within both the reference range and literature range, with the exception of linoleic acid where the means were marginally outside the literature range. The composition of cotton can vary significantly with the site and agricultural conditions, and the differences reported here most likely reflect normal biological variability.

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in cotton line DAS-81910-7 seed when compared with conventional cotton cultivars already available in agricultural markets.

## 7. Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its

consequences, together with an extensive compositional analysis of the food, such as that presented in Section 6 of this report.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock or other animal species will add little to the safety assessment (Bartholomaeus *et al.*, 2013; OECD, 2003). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

Cotton line DAS-81910-7 is the result of a simple genetic modification to confer dual herbicide tolerance, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modification has not altered the nutritional adequacy of cotton line 81910 as a source of food when compared with that of conventional cotton varieties. The introduction of foods derived from cotton line DAS-81910-7 into the food supply is therefore expected to have negligible nutritional impact.

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